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(54) Title: BLOOD-BRAIN BARRIER TRANSPORTERS OF NEUROLOGICAL AGENTS

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ATG GCA GCC GGG AGC ATC ACC ACG CTG CCC GCC TTG CCC GAG GAT GGC
Met Ala Ala Gly Ser Ile Thr Thr Leu Pro Ala Leu Pro Glu Asp Gly
  1               5                  10                  15

GGC AGC GGC GCC TTC CCG CCC GGC CAC TTC AAG GAC CCC AAG CGG CTG
Gly Ser Gly Ala Phe Pro Pro Gly His Phe Lys Asp Pro Lys Arg Leu
            20                  25                  30

TAC TGC AAA AAC GGG GGC TTC TTC CTG CGC ATC CAC CCC GAC GGC CGA
Tyr Cys Lys Asn Gly Gly Phe Phe Leu Arg Ile His Pro Asp Gly Arg
        35                  40                  45

GTT GAC GGG GTC CGG GAG AAG AGC GAC CCT CAC ATC AAG CTA CAA CTT
Val Asp Gly Val Arg Glu Lys Ser Asp Pro His Ile Lys Leu Gln Leu
    50                  55                  60

CAA GCA GAA GAG AGA GGA GTT GTG TCT ATC AAA GGA GTG TGT GCT AAC
Gln Ala Glu Glu Arg Gly Val Val Ser Ile Lys Gly Val Cys Ala Asn
65                  70                  75                  80

CGT TAC CTG GCT ATG AAG GAA GAT GGA AGA TTA CTG GCT TCT AAA TGT
Arg Tyr Leu Ala Met Lys Glu Asp Gly Arg Leu Leu Ala Ser Lys Cys
                85                  90                  95

GTT ACG GAT GAG TGT TTC TTT TTT GAA CGA TTG GAA TCT AAT AAC TAC
Val Thr Asp Glu Cys Phe Phe Phe Glu Arg Leu Glu Ser Asn Asn Tyr
            100                 105                 110

AAT ACT TAC CGG TCA AGG AAA TAC ACC AGT TGG TAT GTG GCA TTG AAA
Asn Thr Tyr Arg Ser Arg Lys Tyr Thr Ser Trp Tyr Val Ala Leu Lys
        115                 120                 125

CGA ACT GGG CAG TAT AAA CTT GGA TCC AAA ACA GGA CCT GGG CAG AAA
Arg Thr Gly Gln Tyr Lys Leu Gly Ser Lys Thr Gly Pro Gly Gln Lys
    130                 135                 140

GCT ATA CTT TTT CTT CCA ATG TCT GCT AAG AGC TGA
Ala Ile Leu Phe Leu Pro Met Ser Ala Lys Ser Ter
145                 150                 155
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(57) Abstract

The invention provides method for inducing neuronal precursor cells (NPCs) in vitro and in vivo by contacting NPC with at least one proliferation factor to promote mitosis, survival and/or differentiation of at least one type of NPC and can be used to treat neuronal cell disorders caused by disease, injury, and other neural disorders, as well as methods for transporting molecules across the blood brain barrier by linking them to portions of at least one growth factor to effectively introduce neurological agents capable or incapable of independently crossing the blood brain barrier into the central nervous system. The figure shows the human form of bFGF.

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"BLOOD-BRAIN BARRIER TRANSPORTERS OF NEUROLOGICAL AGENTS"

Background of the Invention

Field of the Invention

The present invention relates generally to the delivery of neurological agents, such as diagnostic and therapeutic agents, across the blood-brain barrier, using a transport factor as at least one portion of a growth factor.

Description of the Background Art

Neurogenesis

The development of neurons in the brain from precursor cells is a highly complex process known as neurogenesis. Neurogenesis has been studied in the developing brain of both avian and mammalian species (see, e.g., Jacobson, *Developmental Neurobiology*, Plenum Press, New York (1978); Schacher, in *Principles of Neural Science*, Kandel *et al.*, eds., Elsevier/North Holland, NY (1985), pp. 729-742). Data from these studies has been used to partially characterize the timing and structural aspects of neuronal production in cell populations of both the peripheral nervous system (PNS) and the central nervous system (CNS) (see, e.g., Cowan in *Development and Aging in the Nervous System*, Rockstein, ed., Academic Press, NY, (1973), pp. 19-41; Hamburger, *Comp. Neurol. 160:*535-546 (1975); Carr *et al., J. Comp. Neurol. 182:*727-740 (1978); Hamburger *et al., J. Neurosci. 1:*60-71 (1981)).

Although some neuronal cell production occurs in the nervous system in apparent excess, quantitatively (Hamburger, *infra*, (1949); Hamburger,

infra, (1975); Hamburger, *infra*, (1981)), the reproducibility of neuronal generation suggests that the process is precisely regulated (Cowan, *infra*; Frederikson *et al.*, *J. Neurosci. 8:*1144-1151 (1988); Rakic, *Science 241:*170-176 (1988).

Early investigations into the control of neurogenesis examined the role of target organs to which neurons projected *in vivo*, initially suggesting that target-derived factors, such as nerve growth factor (NGF), can influence neuronal production (Hamburger, *infra*, (1949); Prestige, *J. Embryol. Exp. Morphol. 17:*453-471 (1967)). While target derived factors were initially thought to have a mitogenic effect upon these neurons (Levi-Montalcini *et al.*, *Proc. Natl. Acad. Sci. 46:*384-391 (1960); Thoenen, H. *et al.*, *Physiol. Review 60:* 1284-1335 (1980)), studies of systems in which target-dependent survival was temporally distinct from the phase of neuroblast production provided clear evidence that target molecules did not influence neuronal precursor division, but instead promoted neuronal survival (Cowan, *infra*; Landmesser *et al.*, *J. Physiol. (London) 241:* 715-736 (1974); Hamburger *infra*, (1975); Carr, *infra*, (1978); Wright *et al.*, *Int. J. Develop. Neurosci. 5:*305-311 (1987)).

Soon after birth, neuronal production ceases in the mammalian brain, suggesting that a quiescent stem cell population is not maintained, in contrast to most other tissues (Jacobson, *supra*; Schacher, *supra*; Black *et al.*, "Nerve growth factor and the issue of mitosis in the nervous system", in *Current Topics in Developmental Biology 24:* 161-192 (1990)). Therefore, in order to study factors which influence neuronal precursor division, investigators have focused research efforts on fetal or embryonic neural tissue.

In vivo studies have examined the potential mitogenic roles of thyroid and growth hormones, and endogenous monoamine neurotransmitters (LeGrand, in *Brain: Fetal and Infant*, Berenberg, ed., Nijhoff, The Hague (1977), pp. 137-164; Lauder, *Psychoneuroendocrinology 8:*121-155 (1983)). Although normal activity of these agents was found to be required for physiological brain growth, potential direct mitogenic effects were obscured

by the complexities of the maternal-fetal unit and the undefined fetal microenvironment.

As an alternative to *in vivo* systems, tissue culture systems have been developed and used to study the effects of growth factors on neuronal cells derived from the brain. Defined culture systems have been developed for substantially pure populations of neuronal precursor cells from embryonic rat superior cervical ganglion (DiCicco-Bloom *et al.*, *Proc. Natl. Acad. Sci. USA* *85*:4066-4070 (1988); DiCicco-Bloom *et al.*, *J. Cell Biol.* *110*:2073-2086 (1990)) and the external germinal layer (EGL) of the early postnatal mouse cerebellum (Gao *et al.*, *Neuron* *6*:705-715 (1991)). Culture systems have also been developed for populations of pluripotent cells from embryonic forebrain (Temple, *Nature* *340*:471-473 (1989); Anchan *et al.*, *Neuron* *6*:923-936 (1991); Kilpatrick *et al.*, *Neuron* *10*:255-265 (1993)).

In addition, pluripotent cells from adult mouse brain are capable of dividing and differentiating into glial cells and neurons *in vitro* have been successfully cultured (Reynolds *et al.*, *Science* *255*:1707-1710 (1992); Richards *et al.*, *Proc. Natl. Acad. Sci. USA* *89*:8591-8595 (1992); Lois *et al.*, *Proc. Natl. Acad. Sci. USA* *90*:2074-2077 (1993)).

The use of identified neuronal precursor populations in fully defined media is now permitting the identification of some growth factors involved in neuronal precursor cell proliferation. However, the study of growth factor effects on these cultured neuronal precursors is more complex than in nonneuronal cell systems due to the potential for trophic as well as mitogenic effects on neuronal populations. In nonneuronal systems culture conditions ensuring optimal survival have been established, eliminating the possibility of confounding trophic with mitogenic effects.

In contrast, in neuronal precursor cells growth factors can act either as trophic factors, promoting the survival of dividing neuroblasts, or they can act as mitogens, actually stimulating entry into the mitotic cycle, or they can exhibit both activities. It is difficult to distinguish mitogenic and trophic

effects because both categories of action yield apparent evidence of increased mitosis per se using traditional assays.

Fibroblast Growth Factors

Basic fibroblast growth factor (bFGF) is a single chain, non-glycosylated protein which has been isolated from several different tissues in forms of varying molecular weight ranging in length from about 146 to about 155 amino acids, due primarily to differing N-termini. The cloning and sequencing of the gene encoding bFGF from bovine and human sources has been reported (Ersch *et al., Proc. Natl. Acad. Sci. 82*:6507-6511 (1985); Abraham *et al., Science 233*:545-548 (1986); Abraham *et al., EMBO J. 5*:2523-2528 (1986)).

bFGF has been suggested to be involved in the induction of neuronal precursor proliferation *in vitro* (Gensburger *et al., FEBS Letters 217*:1-5 (1987); Murphy *et al., J. Neurosci. Res. 25*:463-475 (1990); Cattaneo *et al., Nature 347*:762-765 (1990); Gao, *infra*; Richards, *infra*). However, no mitogenic effects for bFGF have been demonstrated in the brain *in vivo*.

The Blood Brain Barrier (BBB)

The blood brain barrier (BBB) is primarily composed of specialized capillary endothelial cells that supply blood to the brain. These cells are joined by tight junctions in virtually all vertebrates which effectively restrict the passage of molecules from the bloodstream to the brain tissues and act as the BBB (see Goldstein *et al., Scientific American 255*:74-83 (1986); Partridge, *Endocrine Reviews 7*(3):314-330 (1986)), particularly those which are larger than about 200 daltons, charged, and/or water soluble. The BBB creates a controlled molecular environment in the brain that allows this organ to function normally under a variety of circumstances and protects the brain from deleterious substances in the bloodstream.

Unfortunately, the BBB also excludes the delivery of many useful therapeutic and diagnostic agents *via* the bloodstream. In particular, it has been reported that the BBB may exclude the delivery of systemically administered NGF due to the chemical properties of these factors (see, e.g., Follesa *et al., Mol. Pharmacol. 43*:132-138 (1993)). Thus, there is currently a need for an efficient, noninvasive means for delivering such agents across the BBB.

To deliver such agents across the BBB, some investigators have proposed linking the agent with a carrier, such as a small, lipid soluble molecule (see Bodor, *Ann. N.Y. Acad. Sci. 507*:289-306 (1987)), and insulin, transferrin, and insulin-like growth factors (IGFs) I and II (Pardgridge, *Ann. N.Y. Acad. Sci. 529*:50-60 (1988)). In a related methodology, a transferrin-receptor antibody has been suggested as a carrier protein (see U.S. Patent Nos. 5,154,924 and 5,182,107 by Friden; and Friden *et al., Science 259*:373-377 (1993)).

It has also been determined in the art of brain pharmaceuticals, including therapeutic and diagnostic agents, that at least three criteria, as listed below, must be met in order for a putative brain therapeutic or imaging agent to be found suitable for *in vivo* brain therapeutic or diagnostic administration. These criteria include, but are not limited to, the following.

One criteria is that such agents must pass the blood brain barrier by meeting a lower and upper lipophilic threshold, wherein a neuropharmaceutical must be lipophilic enough to penetrate the blood-brain barrier. Lipid insoluble molecules, with very few exceptions, do not penetrate into the brain. Similarly, the compound cannot be too lipophilic; if it is, it will bind non-specifically to cell membranes, resulting in generalized distribution in the brain, mimicking the action of a blood flow agent as opposed to a specific receptor-binding agent.

Another criteria is metabolic stability of the brain therapeutic or imaging agent after administration. The agent must be sufficiently metabolically stable in the brain to allow for its therapeutic or imaging effects

by binding to the appropriate receptor. A third criteria is the retention of receptor specificity and selectivity of the brain therapeutic or imaging agent for the particular receptor for which it is specific, while at the same time having a high selectivity by having a low binding to other, non-target receptors. For example, the agent must retain its specificity and selectivity at 37°C, which is not always true for compounds tested *in vitro*, where incubation conditions are typically 4°C or at ambient room temperature. It is well-known that affinity and metabolic rate are greatly influenced by temperature. These criteria are presented in the following references: Arendt *et al.*, *Cardiology 71*:307-314 (1984); Garvey *et al.*, *J. Pharmacol. Exp. Ther. 194*:220-233 (1975); Arnett *et al.*, *J. Neurochem. 44*:835 (1985); Arnett *et al.*, *J. Nucl. Med. 27*:1878-1882 (1986); Kung, *Nuc. Med. Biol. 17*:85-92 (1990).

Unless, at a minimum, the above three criteria are met, then a brain therapeutic agent is not shown to be suitable for *in vivo* brain therapy, as showing a correlation with *in vivo* efficacy. For example, if a agent passes the blood brain barrier and binds receptors, but does not have metabolic stability or loses its selectivity or specificity, then such a agent is not suitable for *in vivo* brain therapy.

At present, development of methods to deliver agents across the BBB using carrier compounds is limited by the small number of carrier compounds that have been identified and limitations inherent in these compounds. Other compounds useful as carriers are yet to be identified.

Citation of any document herein is not intended as an admission that such document is pertinent prior art, or considered material to the patentability of any claim of the present application. Any statement as to content or a date of any document is based on the information available to applicant at the time of filing and does not constitute an admission as to the correctness of such a statement.

Summary of the Invention

The present invention is intended to overcome one or more deficiencies of the related background art.

The present invention also provides methods and compositions using transport factors (TFs), which, when administered to an animal, can pass the blood-brain barrier (BBB) and provide a diagnostic and/or therapeutic effect in the central nervous system (CNS). The present invention also provides methods and compositions using TFs which, when associated with a neurological agent and administered to an animal, can transport the agent accross the BBB, such that the agent can elicit at least one diagnostic and/or therapeutic effect in the (CNS).

The present invention further provides methods for inducing the growth and/or proliferation of neuronal precursor cells (NPCs) in a animal by administering at least one proliferation factor (PF) comprising at least a portion of at least one growth factor to that animal. These methods can be applied to treat neurological disease or trauma conditions or pathologies caused by the loss or degeneration of neuronal cells.

Other features, embodiments and utilities of the present invention will be apparent to skilled practitioners from the following detailed description and examples relating to the present invention.

Brief Description of the Drawings

Figure 1 illustrates the DNA coding and amino acid sequence of human basic fibroblast growth factor (*see*, e.g., Abraham *et al.*, *EMBO J.* *5*(10):2523-2528 (1986).

Figure 2 illustrates a graph of tritiated thymidine incorporation by cerebellar granule cells in culture following treatment with bFGF.

Detailed Description of the Invention

Transport Factors for Transport of Neurological Agents Across the Blood Brain Barrier

The present invention is based in part upon the discovery that transport factors (TFs) can cross the blood-brain barrier (BBB) and elicit effects on the brain after administration. The present invention also provides methods for transporting neurological agents across the BBB by linking such agents to a transport factor (TF) which retains the ability to cross the BBB.

As used herein, the term "neurological agent" refers to any molecule which is desired to be introduced into the central nervous system (CNS) but which can be incapable of, or restricted from, independently crossing the BBB, as further described herein.

According to the present invention, a neurological agent can be linked to a transport factor in a manner which does not inhibit the desired activity of the neurological agent in the CNS or the ability of the TF to cross the BBB. Such linkages can be formed using various conjugation/cross-linking techniques well known in the art (*see*, e.g., U.S. Patent No. 5,182,107, issued Jan 26, 1993; Ausubel, *infra*; and Sambrook, *infra*; incorporated entirely by reference herein). These techniques include the formation of permanent linkages as well as linkages susceptible to cleavage. Cleavable linkages can be used in situations where it is desirable for the neurological agent to be separated from the TF after transport across the BBB. Such a separation can be desirable, for example, in situations where the neurological agent is more effective in the CNS in an unlinked form.

Where the neurological agent to be transported is a protein or peptide, linkages with a TF include protein fusions. Fusions of a protein or peptide with a TF can be accomplished using recombinant DNA technology by fusing DNA encoding a TF with DNA encoding the neurological agent. To optimize

the retention of the activity of the fused polypeptides, peptide linkers can be used. Peptide linkers designed to link various polypeptides in a manner which allows the linked polypeptides to retain their activity are well known in the art. *See*, e.g., Ausubel *et al.*, eds, *Current Protocols in Molecular Biology*, Greene Publishing Assoc., New York, NY (1987, 1988, 1989, 1990, 1991, 1992, 1993, 1994) at §§ 1-4, 16; and Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual,* Second Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), at §§1-5, 16-18; and Huston, J.S. *et al.*, *Methods Enz. 203*:46-88 (1991).

A TF-neurological agent conjugate can be administered *in vivo* for delivery across the blood brain barrier (BBB) by any method known to the skilled artisan. TFs having transport activity, such as proliferative factors (TFs) which exhibit trophic or mitogenic effects on cell culture populations, are thus useful in the methods of the present invention to transport neurological agents across the blood brain barrier.

Transport Factors (TFs)

Transport factors (TFs) which are useful in the present invention include at least one TF which has transport activity by being capable of passing the BBB alone, or in combination with a neurological agent. Preferably, a TF of the present invention is related in structure and function to at least a portion of at least one "growth factor." The term "growth factor," as used herein, is intended to cover any protein, peptide, molecule or portion thereof, which is capable of promoting, directly or indirectly, at least one of the survival, proliferation, and/or differentiation of a neurological cell type, including neuronal precursor cells (NPCs).

Such growth factors include, but are not limited to, at least one selected from the group consisting of a nerve growth factor (NGF), a brain derived neurotrophic factor (BDNF), a neurotrophin-3 (NT3), a neurotrophin-4 (NT4), a neurotrophin-5 (NT5), a fibroblast growth factor (FGF), a

transforming growth factor (TGF), a glial derived neurotrophic factor (GDNF), an epidermal growth factor (EGF), a platelet derived growth factor (PDGF), an insulin, an insulin-like growth factor (IGF), a vasoactive intestinal peptide (VIP), a pituitary adenylate cyclase activiating polypeptide (PACAP), a human chorionic gonadotrophin (hCG), an interferon, a leukemia inhibitory factor (LIF, also known as cholinergic differentiation factor (CDF) and D factor), a calcitonin gene related peptide (CGRP), a vasopressin, a bradykinin, a bombesin, a Substance P, an enkephelin, an interferon, a somatostatin and an interleukin.

FGFs are preferred growth factors. As used herein, the term "fibroblast growth factor" ("FGF") is intended to refer to any form of of a fibroblast growth factor. As a non-limiting example, βGFG is a single chain, non-glycosylated protein which has been isolated from several different tissues in forms of varying molecular weight ranging in length from about 146 to about 155 amino acids due primarily to differing N-termini. For use in the methods described herein, the preferred form of bFGF is that form derived from the species and/or subject to which the methods are applied. In particular, the human form of bFGF is shown in Figure 1, e.g., as described in Abraham *et al., EMBO J. 5*:2523-2528 (1986), as the form preferred for use in humans.

Other forms of FGFs are well known in the art. Fibroblast growth factors (FGFs) also include, but are not limited to, acidic FGF, (Int-2), (hst/K-FGF), FGF-5, FGF-6 and keratinocyte growth factor (KGF). *See*, e.g., Kelley, ed., *Cytokines of the Lung*, Marcel Dekker, Inc., New York (1992), pp. 41-76; Basilico *et al., Advances in Cancer Research 59*:115-165 (1992); Mansukhani *et al, Proc. Nat'l Acad. Sci. USA 89*:3305-3309 (1992); Rifkin *et al., J. Cell Biology 109*:1-6 (1989); U.S. Patent Nos. 4,296,100, 4,378,347, 4,642,120; EP Publications EP 281 822 and EP 320 148, the disclosure of which references are entirely incorporated herein by reference.

Cytokines are protein growth factors that function as intercellular signals that regulate local and/or systemic metabolic responses, which include

peptides and interleukins having such activity. Interleukins were initially discovered and defined as proteins produced by leukocytes that affect leukocytes or other target cells. An interleukin designation is assigned to any protein that fulfills this definition. However, additional proteins fit the classification as an interleukin, but were named before this classification, such that the term cytokine is now used as the general term.

Known cytokines include **interleukins** (IL) IL-1 (also endogenous pyrogen (EP), lymphocyte activating factor (LAF), mononuclear cell factor, catabolin, osteoclast activating factor and hematopoetin 1), IL-2 (also T cell growth factor (TCGF)), IL-3 (multicolony stimulating factor (M-CSF), P-cell stimulating factor, WEHI-3B factor, mast-cell growth factor and histamine-producing factor), IL-4 (B-cell growth factor (BCGF), B-cell stimulatory factor-1 (BSF-1), IL-5 (T-cell replacing factor (TRF), B-cell growth factor II (BCGF-II), eosinophil differentiation factor (EDF), IL-6 (β_2 interferon (IFN-β_2), B-cell stimulating factor 2 (BSF-2), 26-kDa protein, hybridoma/plasmacytoma growth factor (HPGF or IL-HP-2), hepatocyte stimulating factor (HSF), and T-cell activating factor (TAF)), IL-7, IL-8 (neutrophil activating protein 1 (NAP-1), IL-10 (also cytokine synthesis inhibitory factor (CSIF); **tissue necrosis factors** (TNF) TNFα (also lymphotoxin (LT) and TNFβ (also macrophage derived TNF); **inteferons** (IFN) IFNα and IFNβ (also type I IFN) and IFNγ (also type II IFN) and **tissue growth factor (TGF)** β.

See, e.g., Dawson, in *Lymphokines and Interleukins*, Dawson, ed., CRC Press, Boca Raton, Florida (1991); Mosmann *et al.*, *Immunol. Rev.* *123*:209-229 (1991); Mosmann *et al.*, *Immunol. Today 12*:A59-A69 (1991); Sherry *et al.*, *Curr. Opinion Immunol. 3*:56-60 (1991); Paul, *Blood 77*:1859-1870 (1991); Dower *et al.*, *J. Clin. Immunol. 10*:289-299 (1990), which references are entirely incorporated herein by reference.

Accordingly, a TF of the present invention alternatively includes one or more polypeptides having a sequence which substantially corresponds to at least one 3 to 500 amino acid fragment, variant and/or consensus sequence of

a known growth factor or group of growth factors, wherein the TF has a sequence having significant homology or identity to a corresponding fragment or consensus sequence, e.g., having at least 80%, such as 80-99% homology, or any range or value therein. A TF of the present invention is not naturally occurring or is naturally occurring but is provided according to the present invention in a purified or isolated from which does not occur in nature. Preferably, a TF of the present invention substantially corresponds to at least one mitotic/trophic active portion of a growth factor, variant or consensus sequence of a growth factor selected from the group consisting of an insulin, an insulin-like growth factor (IGF), a vasoactive intestinal peptide (VIP), a pituitary adenylate cyclase activating polypeptide (PACAP), an interferon, a neurotrophin-3 (NT3), a nerve growth factor (NGF), a fibroblast growth factor (FGF), a ciliary neurotrophic factor (CNTF), a brain derived neurotrophic factor (BDNF), and somatostatin, depending on the NPC cell type or preferred mitotic or trophic effect desired in the particular application of a method of the present invention, as described herein.

Percent homology or identity can be determined, e.g., by comparing sequence information using the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol. 48*:443 (1970)), as revised by Smith and Waterman (*Adv. Appl. Math. 2*:482 (1981)). Briefly, the GAP program defines similarity as the number of aligned symbols (i.e., nucleotides or amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res. 14*:6745 (1986), as described by Schwartz *et al.*, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation (1979), pp. 353-358; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

Thus, one of ordinary skill in the art, given the teachings and guidance presented in the present specification, will know how to substitute other amino acid residues in other positions of a growth factor sequence to obtain a TF, including substituted, deletional or insertional variants.

A TF of the present invention thus also can include at least one variant growth sequence having mitotic/trophic activity on at least one NPC type, wherein at least one amino acid residue in the polypeptide has been conservatively replaced, inserted or deleted by at least one different amino acid, such as 1, 2, 3, 4, 5, 6, 7, 8,, 9, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70 80, 90, or 100, or any range or value therein.

An amino acid or nucleic acid sequence of a TF of the present invention is said to "substantially correspond" to another amino acid or nucleic acid sequence respectively, if the sequence of amino acids or nucleic acid in both molecules provides polypeptides having biological activity that is substantially similar, qualitatively or quantitatively, to the corresponding fragment of at least one growth factor functional domain. Such "substantially corresponding" TF sequences include conservative amino acid or nucleotide substitutions, or degenerate nucleotide codon substitutions, wherein individual amino acid or nucleotide substitutions are well known in the art.

Accordingly, TFs of the present invention, or nucleic acids encoding therefor, include a finite set of substantially corresponding sequences as substitution peptides or polynucleotides which can be routinely obtained by one of ordinary skill in the art, without undue experimentation, based on the teachings and guidance presented herein. For a detailed description of protein chemistry and structure, see Schulz *et al.*, *Principles of Protein Structure*, Springer-Verlag, New York (1978), and Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco (1983), which are entirely incorporated herein by reference. For a presentation of nucleotide sequence substitutions, such as codon preferences, see Ausubel *et al.*, eds, *Current Protocols in Molecular Biology*, Greene Publishing Assoc., New York, NY (1987, 1988, 1989, 1990, 1991, 1992, 1993, 1994) at §§ A.1.1-

A.1.24, and Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual,* Second Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), at Appendices C and D.

Such substitutions can be made in accordance with the following list, as presented in Table 1, which substitutions can be determined by routine experimentation to provide modified structural and functional properties of a synthesized TF, while maintaining NPC mitotic/trophic biological activity, as determined by at least one suitable activity assay. In the context of the present invention, the term "TF" or "substantially corresponding to" includes such substitutions.

Table 1

Original Residue	Exemplary Substitution
Ala	Gly;Ser
Arg	Lys
Asn	Gln;His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala;Pro
His	Asn;Gln
Ile	Leu;Val
Leu	Ile;Val
Lys	Arg;Gln;Glu
Met	Leu;Tyr;Ile
Phe	Met;Leu;Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp;Phe
Val	Ile;Leu

Alternatively, another group of substitutions of TFs of the present invention are those in which at least one amino acid residue in the protein

molecule has been removed and a different residue inserted in its place according to the following Table 2. The types of substitutions which can be made in the protein or peptide molecule of the present invention can be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz *et al.*, *infra*. Based on such an analysis, alternative conservative substitutions are defined herein as exchanges within one of the following five groups:

TABLE 2

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr (Pro, Gly);
2. Polar, negatively charged residues and their amides: Asp, Asn, Glu, Gln;
3. Polar, positively charged residues:
   His, Arg, Lys;
4. Large aliphatic, nonpolar residues:
   Met, Leu, Ile, Val (Cys); and
5. Large aromatic residues: Phe, Tyr, Trp.

The three amino acid residues in parentheses above have special roles in protein architecture. Gly is the only residue lacking any side chain and thus imparts flexibility to the chain. This, however, tends to promote the formation of secondary structure other than α-helical. Pro, because of its unusual geometry, tightly constrains the chain. It generally tends to promote β-turn-like structures, although in some cases Cys can be capable of participating in disulfide bond formation which is important in protein folding. Note the Schulz et al. would merge Groups 1 and 2, above. Note also that Tyr, because of its hydrogen bonding potential, has significant kinship with Ser and Thr, etc.

Conservative amino acid substitutions, included in the term "substantially corresponding" or "corresponding", according to the present invention, e.g., as presented herein, are well known in the art and would be expected to maintain biological and structural properties of the polypeptide after amino acid substitution. Most deletions and insertions, and substitutions according to the present invention are those which do not produce radical changes in the characteristics of the protein or peptide molecule. "Characteristics" is defined in a non-inclusive manner to define both changes

in secondary structure, e.g., α-helix or β-sheet, as well as changes in physiological activity, e.g., in corresponding activity assays.

However, when the exact effect of the substitution, deletion, or insertion is to be confirmed, one skilled in the art will appreciate that the effect of the substitution, or substitutions, will be evaluated by routine proliferation activity screening assays, either immunoassays or bioassays, to confirm biological activity, such as, but not limited to, trophic and/or mitotic activity on at least one type of NPC.

Amino acid sequence insertions as included in a TF variant of the present invention can also include amino and/or carboxyl-terminal fusions of from 1-100 amino acid residues, or any range or value therein, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions can range generally from about 1 to 30 residues or any range or value therein, more preferably 1 to 5. An example of a terminal insertion includes a fusion with a signal sequence, whether heterologous or homologous to the host cell, to the N-terminus of a TF, to facilitate secretion from recombinant bacterial or eukaryotic hosts. For a detailed description of protein chemistry and structure, see Schulz *et al.*, *Principles of Protein Structure*, Springer-Verlag, New York (1978); and Ausubel, *infra*, which are entirely incorporated herein by reference.

Most deletions, insertions, and substitutions of TFs according to the present invention are those which maintain or improve the mitotic/trophic characteristics of the TF molecule. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. For example, a variant made by site-specific mutagenesis of the peptide molecule-encoding nucleic acid and expression of the variant TF in cell culture or, alternatively, by chemical synthesis, can be tested for NPC mitotic/trophic activity (e.g., as is known in the art and/or as described herein).

Modifications of peptide properties, such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation or the tendency to aggregate with carriers or into multimers, can also be assayed by methods well known to the ordinarily skilled artisan.

Also included in the scope of the present invention are salts of the TFs and factor compositions of the present invention. As used herein, the term "salts" can include, but is not limited to, both salts of carboxyl groups and to acid addition salts of amino groups of the protein or peptide molecule.

Amino acid sequence variants of a TF of the present invention can also be prepared by mutations in the encoding DNA. Such variants include, e.g., deletions from, or insertions or substitutions of, residues within the amino acid sequence. Any combination of deletion, insertion, and substitution can also be made to arrive at the final construct, provided that the final construct possesses some NPC mitotic/trophic activity. Preferably, improved NPC mitotic/trophic activity is found over that of the non-variant peptide. Obviously, mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure (see, e.g., EP Patent Application Publication No. 75,444; Ausubel, *infra*; Sambrook, *infra*).

At the genetic level, such TF variants ordinarily are prepared by site-directed mutagenesis of nucleotides in the DNA encoding a TF or a growth factor, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. The variants can exhibit the same or additional qualitative NPC mitotic/trophic biological activity as the naturally occurring growth factor (see, e.g., Ausubel, *infra*; Sambrook, *infra*).

Knowledge of the three-dimensional structures of proteins is crucial in understanding how they function. The three-dimensional structures of only several thousand proteins is currently available in protein structure databases (in contrast to the more than 200,000 known sequences of proteins and peptides in sequence databases, e.g., Genbank, Chemical Abstracts

REGISTRY, etc.). Analysis of these three-dimensional structures shows that they fall into recognizable classes or motifs. It is possible to model the three-dimensional structure of a protein based on homology to a related protein of known structure. Examples are known where two proteins that have relatively low sequence homology, but are found to have almost identical three dimensional structure. Such homologous variants are also included in TFs of the present invention.

Once a TF structure or characteristic has been determined using the above analysis, TFs can be recombinantly or synthetically produced, or optionally purified, to provide commercially useful amounts of TFs for use in diagnostic or research methods of the present invention, according to the teaching and guidance presented in the present specification, in combination with known method steps, see, e.g., Ausubel, *infra*, and Sambrook, *infra*, which references are herein entirely incorporated by reference.

Recombinant Cloning and/or Production of TFs

Known method steps for synthesizing oligonucleotides probes useful for cloning and expressing DNA encoding a TF of the present invention, based on the teaching and guidance presented herein, are disclosed by, e.g., Ausubel, *infra*, Sambrook, *infra*, and Wu, R., *et al.*, *Prog. Nucl. Acid. Res. Molec. Biol. 21*:101-141 (1978)), which references are entirely incorporated herein by reference.

A suitable oligonucleotide, or set of oligonucleotides, which is capable of encoding (or which is complementary to a sequence encoding) a portion of a NPC mitotic/trophic active growth factor encoding nucleic acid is identified as above, synthesized, and hybridized by means well known in the art, against a DNA, an RNA, or more preferably, a cDNA preparation, derived from cells which are capable of expressing a growth factor gene. Single stranded oligonucleotide probes complementary to an NPC mitotic/trophic activity

encoding sequence can be synthesized using known method steps (see, e.g., Ausubel, *infra*; and Sambrook, *infra*).

Such a labeled, detectable probe can be used by known procedures for screening a genomic or cDNA library, or as a basis for synthesizing PCR probes for amplifying a cDNA generated from an isolated RNA encoding the target nucleic acid or amino acid sequence. As a further non-limiting example, transformants can be selected for expression by a host cell of a target protein, by use of selection media appropriate to the vector used, RNA analysis or by the use of antibodies specific for a target growth factor as a basis for providing a TF used in a method according to the present invention.

A target, detectably labeled probe of this sort can be a fragment of an oligonucleotide that is complementary to a polynucleotide encoding a target protein, as a growth factor. Alternatively, a synthetic oligonucleotide can be used as a target probe which is preferably at least about 10 nucleotides in length (such as 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or more, or any combination or range therein, in increments of 1 nucleotide), in order to be specific for a target a nucleic acid to be detected, amplified or expressed. The probe can correspond to such lengths of a DNA or RNA encoding a growth factor, wherein the probe sequence is selected according to the host cell containing the DNA, e.g., as presented in Table A1.4 of Ausubel *et al.*, *infra*. TF encoding nucleic acids of the present invention can include 9-1500, such as 9-1000, 15-900, 30-200, and 50-500 nucleotides, or any range or value therein, substantially complementary to a portion of at least one growth factor, variant or consensus sequence thereof, wherein codons can be substituted by codons encoding the same or conservatively substituted amino acids, as well known in the art.

Nucleic acids, or protein encoded thereby, to be detected by a method of the present invention, can be contained in samples isolated from any tissue sample of an animal subject or patient, such as blood, lymph, saliva, urine, CNS, amniotic fluid, skin, hair, or any other tissue, and analyzed by

hybridization to labeled probes. Such probes preferably hybridize to target protein-encoding nucleotides under high, moderate or medium stringency conditions, depending on the presense or possible presence of other non-target nucleic acids which also bind the probes specific for the target nucleic acids. For probe design, hybridization, and stringency conditions, see, e.g., Ausubel *supra,* sections 6.3 and 6.4, and Sambrook *et al.*, *supra*. Additional approaches to probe design and detection can also be used, e.g., ligase-mediated gene detection (LMGD), as disclosed, e.g., by Landegren *et al.*, *Science 241*:1077-80 (1988) and fluorescence resonance energy transfer (FRET), as disclosed, e.g., by Wolfe *et al.*, *Proc. Nat. Acad. Sci. USA 85*:8790-94 (1988). See, e.g., Ausubel, *infra*, at §§9.5.2 (selectable markers), §9.8 (RNA analysis), §§10.6-8 (detection of proteins), §§11.1-1.2 (immunoassays) and §§11.3-11.16 (preparation and use of monoclonal, polyclonal and antipeptide antibodies for protein detection).

Culturing of a host and induction of protein expression can be furnished by known methods. A nucleic acid sequence encoding a TF of the present invention can be recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are disclosed, e.g., by Ausubel, *infra*; Sambrook, *infra*, and are well known in the art.

A nucleic acid molecule, such as an RNA or DNA, is said to be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and/or translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression a TF in recoverable amounts. The precise nature of the regulatory regions needed for gene expression can vary

from organism to organism, as is well known in the analogous art. See, e.g., Sambrook, *infra* and Ausubel, *infra*.

The present invention accordingly encompasses the expression of a TF in either prokaryotic or eukaryotic cells, although eukaryotic expression is preferred. Preferred hosts are bacterial or eukaryotic hosts including bacteria, yeast, insects, fungi, bird and mammalian cells either *in vivo*, or *in situ*, or host cells of mammalian, insect, bird or yeast origin. It is preferred that the mammalian cell or tissue is of human, primate, hamster, rabbit, rodent, cow, pig, sheep, horse, goat, dog or cat origin, but any other mammalian cell can be used.

Further, by use of, e.g., the yeast ubiquitin hydrolase system, *in vivo* synthesis of ubiquitin-transmembrane polypeptide fusion proteins can be accomplished. The fusion proteins so produced can be processed *in vivo* or purified and processed *in vitro*, allowing synthesis of a TF of the present invention with a specified amino terminus sequence. Moreover, problems associated with retention of initiation codon-derived methionine residues in direct yeast (or bacterial) expression can be avoided. Sabin *et al.*, *Bio/Technol.* 7(7):705-709 (1989); Miller *et al.*, *Bio/Technol.* 7(7):698-704 (1989).

Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeast are grown in mediums rich in glucose can be utilized to obtain TFs of the present invention. Known glycolytic genes can also provide very efficient transcriptional control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase gene can be utilized.

Production of TFs in insects can be achieved, e.g., by infecting the insect host with a baculovirus engineered to express transmembrane polypeptide by methods known to those of skill. See Ausubel, *infra*, at §§16.8-16.11.

In a preferred embodiment, the introduced nucleotide sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors can be employed for this purpose. See, e.g., Ausubel *et al.*, *infra*, at §§ 1.5, 1.10, 7.1, 7.3, 8.1, 9.6, 9.7, 13.4, 16.2, 16.6, and 16.8-16.11. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector can be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Preferred prokaryotic vectors known in the art include plasmids such as those capable of replication in *E. coli* (such as, e.g., pBR322, ColE1, pSC101, pACYC 184, πVX). Such plasmids are, e.g., disclosed by Sambrook, *infra*; Ausubel, *infra*. *Bacillus* plasmids include pC194, pC221, pT127, etc. Such plasmids are disclosed by Gryczan (In *The Molecular Biology of the Bacilli*, Academic Press, NY (1982), pp. 307-329). Suitable *Streptomyces* plasmids include pIJ101 (Kendall *et al.*, *J. Bacteriol.* *169*:4177-4183 (1987)), and streptomyces bacteriophages such as φC31 (Chater *et al.*, in *Sixth International Symposium on Actinomycetales Biology*, Akademiai Kaido, Budapest, Hungary (1986), pp. 45-54). *Pseudomonas* plasmids are reviewed by John *et al.* (*Rev. Infect. Dis.* *8*:693-704 (1986)); Sambrook, *infra*; and Ausubel, *infra*.

The methods of the present invention can be used to induce the proliferation of neuronal precursor cells in culture. In order to maintain these cells in their undifferentiated state, factors which can restrict the differentiation of these cells, such as the Id protein (*see*, e.g., Duncan *et al.*, *Develop. Biol.* *154*:1-10 (1992)), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF), can be added to the cultures.

Neuronal precursor cultures propagated according to the methods of the present invention can be used, e.g., in various assays to determine the effects of various factors and conditions on neuronal precursor cells.

Neurological Agents

Neurological agents which can be transported across the BBB according to the present invention include, but are not limited to, proteins and small peptides, hormones, antibiotics, nucleotides and nucleotide analogues, drugs, and small molecules. The neurological agent can also be, but is not limited to, at least one therapeutic and/or diagnostic selected from a nucleic acid, a compound, a protein, an element, a lipid, an antibody, a saccharide, an isotope, a carbohydrate, an imaging agent, a lipoprotein, a glycoprotein, an enzyme, a detectable probe, and antibody or fragment thereof, or any combination thereof. For diagnostic uses the neurological agent can be detectably labeled, e.g., as for labeling antibodies, as described herein. Such labels include, but are not limited to, enzymatic labels, radioisotope or radioactive compounds or elements, fluorescent compounds or metals, chemiluminescent compounds and bioluminescent compounds. Alternatively, any other known neurological agent can be used in a method of the present invention.

A neurological agent used in the present invention can have a therapeutic effect on the target cell in the CNS, the effect selected from, but not limited to: correcting a defective gene or protein, a drug action, a toxic effect, a growth stimulating effect, a growth inhibiting effect, a metabolic effect, a catabolic affect, an anabolic effect, an antiviral effect, an antibacterial effect, a hormonal effect, a neurohumoral effect, a cell differentiation stimulatory effect, a cell differentiation inhibitory effect, a neuromodulatory effect, an antineoplastic effect, an anti-tumor effect, an insulin stimulating or inhibiting effect, a bone marrow stimulating effect, a pluripotent stem cell stimulating effect, an immune system stimulating effect, and any other known therapeutic effects that can be provided by a neurological agent delivered to a chimeric receptor cell via a TF according to the present invention.

A therapeutic nucleic acid as a therapeutic agent can have, but is not limited to, at least one of the following therapeutic effects on a chimeric

receptor cell: inhibiting transcription of a DNA sequence; inhibiting translation of an RNA sequence; inhibiting reverse transcription of an RNA or DNA sequence; inhibiting a post-translational modification of a protein; inducing transcription of a DNA sequence; inducing translation of an RNA sequence; inducing reverse transcription of an RNA or DNA sequence; inducing a post-translational modification of a protein; transcription of the nucleic acid as an RNA; translation of the nucleic acid as a protein or enzyme; and incorporating the nucleic acid into a chromosome of a target cell for constitutive or transient expression of the therapeutic nucleic acid.

Therapeutic effects of therapeutic nucleic acids can include, but are not limited to: turning off a defective gene or processing the expression thereof, such as antisense RNA or DNA; inhibiting viral replication or synthesis; gene therapy as expressing a heterologous nucleic acid encoding a therapeutic protein or correcting a defective protein; modifying a defective or underexpression of an RNA such as an hnRNA, an mRNA, a tRNA, or an rRNA; encoding a toxin in pathological cells; encoding a drug or prodrug, or an enzyme that generates a compound as a drug or prodrug in pathological or normal cells expressing the chimeric receptor; encoding a thymidine kinase varicella-zoster virus thymidine kinase (VZV TK) (see, e.g., Huber et al *Proc. Nat'l Acad. Sci. USA 88*:8039-8042 (1992), the entire contents, including cited references, are entirely incorporated by reference) in pathogenic cells, such as neoplastic cells to directly or indirectly kill such pathogenic cells; and any other known therapeutic effects. Mutant nucleic acids such as those described above can thus be used in gene therapy or other therapy in the CNS.

A therapeutic nucleic acid of the present invention which encodes, or provides the therapeutic effect of any known toxin, prodrug or drug gene for delivery to pathogenic cells can also include genes under the control of a tissue specific transcriptional regulatory sequence (TRSs) specific for pathogenic cells, such as neoplastic cells, including α-fetoprotein TRS or liver-associated albumin TRS (see, e.g., Dynan & Tjian *Nature 316*:774-778

(1985)). Such TRSs would further limit the expression of the cell killing toxin, drug or prodrug in the target cell.

It is understood that the dosage of a neurological agent of the present invention administered *in vivo* or *in vitro* will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the diagnostic/ therapeutic effect desired. The ranges of effective doses provided herein are not intended to be limiting and represent preferred dose ranges. However, the most preferred dosage will be tailored to the individual subject, as is understood and determinable by one skilled in the relevant arts.

The total dose required for each treatment can be administered by multiple doses or in a single dose. The neurological agent can be administered alone or in conjunction with other diagnostics and/or therapeutics directed to the pathology, or directed to other symptoms of a CNS pathology.

Effective amounts of a neurological agent of the present invention are from about 0.001 μg to about 100 mg/kg body weight, and preferably from about 1 μg to about 50 mg/kg body weight.

In Vivo Proliferation Factors (PFs)

The present invention is based in part on the discovery that **proliferation factors (PFs)** can induce mitosis and/or survival of neuronal precursor cells (NPCs) of the brain *in vivo* and *in vitro*.

According to the present invention, methods and compositions are also provided for inducing at least one type of neuronal precursor cells (NPCs) to proliferate by providing to such cells PFs, compositions, and methods capable of promoting proliferation including both NPC division (i.e., a mitogen inducing mitosis) and NPC survival (i.e., a trophic factor inducing trophism). A proliferative composition useful in these methods can comprise a single proliferation factor (PF) with both mitogenic and trophic capabilities.

Alternatively or additionally, a PF of the present invention can comprise a mitogenic factor and/or a trophic factor.

In *in vivo* or *in vitro* environments where at least one type of NPC is in the presence of a mitogenic factor capable of promoting their division, the present invention provides methods and compositions for inducing proliferation of these NPCs by providing to such cells PFs capable of promoting the NPC's survival (i.e., trophic factors). Alternatively, in *in vivo* or *in vitro* environments, where the neuronal precursor cells are in the presence of a trophic factor capable of promoting their survival, the present invention provides methods for inducing the proliferation of these cells by providing to such cells proliferation factors capable of promoting their division (i.e., mitogenic factors).

Proliferative factors which are useful in the present invention include those which act generally as mitogens and/or trophic factors for one or more of a variety of NPC types, as well as those factors whose effects are specific to a particular neuronal precursor type or developmental stage, or those whose effects vary (i.e., factors which can act as a mitogen or trophic factor depending upon the cell type and developmental stage).

Factors with both mitogenic and trophic capabilities and chimeric proteins consisting of fusions of a trophic factor and a mitogen can also be used in the methods of the present invention. Additional proliferation factors with mitogenic and/or trophic capacities useful in the methods of the present invention can be identified based upon their ability to increase cell division and/or survival of the neuronal precursor cell type whose proliferation is desired to be induced.

One source of such additional PFs is the group of polypeptides known to promote the survival and/or differentiation of neuronal cells which are referred to herein as "growth factors". The term "growth factor," as used herein, is intended to cover any protein, peptide, molecule or portion thereof, which is capable of promoting, directly or indirectly, at least one of the survival, proliferation, and/or differentiation of a given cell type. Such

growth factors include, but are not limited to, those described above for transport factors.

The survival promoting effect of growth factors upon neuronal cells is referred to herein as a "trophic effect." The term "mitosis" as used herein refers generally to the process of division a cell into two or more daughter cells. Ther term "differentiation" as used herein refers generally to the process of one or more stages of development of a cell from a less differentiated form to a more differentiated form, usually wherein fewer genes are available for expression. For neuronal cells, differentiation typically includes an increase in the size of the neuronal cell and an increase in the complexity of the shape of the neuronal cell, morphological changes which can be monitored as a marker for differentiation. The term "differentiation-promoting effect" as used herein refers to the ability of a proliferation or growth factor to promote the process of neuronal cell differentiation.

According to the methods of the present invention, the mitosis of neuronal precursor cells can be induced *in vivo* or *in vitro* by providing PFs, including, for example, mitogens, such as bFGF, to the brain. NPCs responsive to these methods can include, but are not limited to, those cells which possess receptors for mitogenic compounds, such as bFGF (see Wanaka, A. *et al., Development 111*:455-468 (1991); Fayein, N-A. *et al., Biol. Cell. 76*:1-13 (1992)) and include, but are not limited to, cerebellar granule precursors, hippocampal precursors, precursors in the embryonic ventricular zone neuroepithelium, and sympathetic and dorsal root ganglia.

NPCs capable of dividing and differentiating into neuronal cells have now been detected in the adult brain (see Reynolds *et al., Science 255*:1707-1710 (1992); Richards *et al., Proc. Natl. Acad. Sci. USA 89*:8591-8595 (1992); Lois *et al., Proc. Natl. Acad. Sci. USA 90*:2074-2077 (1993)). Thus the methods provided are contemplated to be useful for inducing neuronal precursor mitosis not only in the brain during early developmental stages, but throughout development and into adulthood.

PFs can be identified as mitogenic and/or trophic factors for NPCs by utilizing assays which distinguish increased cell proliferation due to increased cell division from increased cell proliferation due to increased survival of dividing cells. Precursor populations are composed of both mitotic cells (undergoing division) and non-mitotic cells. Mitotic cells can be detected by incorporation of labeled nucleotides, such as (^{3}H)-thymidine, into genomic DNA. Increased DNA synthesis in a population can reflect an increased proportion of the cells undergoing mitosis or increased survival of mitotic cells in an *in vitro* culture system or an *in vivo* assay system. Importantly, mitotic precursors in populations fail to survive in these assays. Mitogenic effects can be identified by an increase in the ratio of mitotic cells to total cells in the population, visualized by autoradiography to detect radiolabeled nucleotide in nuclear DNA, under particular temporal culture conditions (see DiCicco-Bloom *et al.*, *Proc. Natl. Acad. Sci. USA 85*:4066-4070 (1988); DiCicco-Bloom *et al.*, *J. Cell Biol. 110*:2073-2086 (1990)). This ratio is referred to as the labeling index (LI). Mitogens increase the LI. Increased survival can be detected by monitoring the number of cells present initially and at later time points, such as 8, 24 or 48 hours. Trophic factors are those which induce an increase in total cell number but do not cause a concomitant increase in the LI, typically yielding little or no change in the LI and possibly even a decrease (see examples provided herein, DiCicco-Bloom *et al.*, *Proc. Natl. Acad. Sci. USA 85*:4066-4070 (1988); DiCicco-Bloom *et al.*, *Society for Neuroscience Abstracts XV*:328 (1989); Chen *et al.*, *Society for Neuroscience Abstracts XVI*:803 (1990); Cohen *et al.*, *Society for Neuroscience Abstracts XVI*:804 (1990); DiCicco-Bloom *et al.*, *J. Cell Biol. 110*:2073-2086 (1990)). Further methods include prelabeling the mitotic cells with (^{3}H)-thymidine at plating and documenting whether they survive at later times or divide to produce a labeled doublet of cells. Alternatively, time-lapse photomicroscopy can be employed to define the mitotic and survival fate of precursors (see, e.g., Example 7; DiCicco-Bloom (1990), *supra*).

A proliferative factor (PF), according to the present invention, refers to a factor comprising at least one portion of a growth factor having at least one of mitotic and trophic activity on at least one type of neuronal precursor cell (NPC), such as cerebellar granular precursors, neuroblasts and telencephalic precursor cells. A growth factor portion according to the present invention can be prepared by proteolytic digestion of an intact growth factor molecule or a fragment thereof, by chemical peptide synthesis methods well-known in the art, by recombinant DNA methods discussed in more detail below, and/or by any other method capable of producing a PF and having a conformation similar to an active portion of at least one growth factor having at least one of mitotic or trophic activity on at least one NPC, according to a suitable screening assay, e.g., as described herein and/or as known in the art. The minimum peptide sequence to have mitotic and/or trophic activity is based on the smallest unit containing or comprising a particular region, consensus sequence, or repeating unit thereof of a growth factor having mitotic/trophic activity.

Accordingly, a PF of the present invention alternatively includes one or more polypeptides having a sequence which substantially corresponds to at least one 3 to 500 amino acid fragment, variant and/or consensus sequence of a known growth factor or group of growth factors, wherein the PF has a sequence having significant homology or identity to a corresponding fragment or consensus sequence, e.g., having at least 80%, such as 80-99% homology, or any range or value therein. A PF of the present invention is not naturally occurring or is naturally occurring but is provided according to the present invention in a purified or isolated from which does not occur in nature. Preferably, a PF of the present invention substantially corresponds to at least one mitotic/trophic active portion of a growth factor, variant or consensus sequence of a growth factor, as described herein for transport factors (TFs).

Once a PF structure or characteristic has been determined using the above analysis, PFs can be recombinantly or synthetically produced, or optionally purified, to provide commercially useful amounts of PFs for use in

diagnostic or research methods of the present invention, according to the teaching and guidance presented in the present specification, in combination with known method steps, see, e.g., Ausubel, *infra*, and Sambrook, *infra*, which references are herein entirely incorporated by reference.

Recombinant Cloning and/or Production of PFs

Known method steps for synthesizing oligonucleotides probes useful for cloning and expressing DNA encoding a PF of the present invention, based on the teaching and guidance presented herein, are disclosed by, e.g., Ausubel, *infra*, Sambrook, *infra*, and Wu, R., *et al.*, *Prog. Nucl. Acid. Res. Molec. Biol. 21*:101-141 (1978)), which references are entirely incorporated herein by reference. Cloned and/or expressed PFs can be made and used according to known method steps, similarly to the TFs, as described herein.

The methods of the present invention can be used to induce the proliferation of NPCs *in vitro*, e.g., in culture. In order to maintain these cells in their undifferentiated state, factors which can restrict the differentiation of these cells, such as the Id protein (*see*, e.g., Duncan *et al.*, *Develop. Biol. 154*:1-10 (1992)), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF), can be added to the cultures.

Neuronal precursor cultures propagated according to the methods of the present invention can be used, e.g., in various assays to determine the effects of various factors and conditions on neuronal precursor cells.

Therapeutic Applications

Pharmaceutical administration of a PF, TF or TF-neurological agent, e.g., as a compound or composition of the present invention can be administered by any means that achieve its intended purpose, for example, to treat or prevent a neurological disorder.

The term "protection," as in "protection from infection or disease," as used herein, encompasses "prevention," "suppression" or "treatment." "Prevention" involves administration of a Pharmaceutical composition *prior to the induction* of the disease. "Suppression" involves administration of the composition *prior to the clinical appearance* of the disease. "Treatment" involves administration of the protective composition *after the appearance* of the disease. It will be understood that in human and veterinary medicine, it is not always possible to distinguish between "preventing" and "suppressing" since the ultimate inductive event or events can be unknown, latent, or the patient is not ascertained until well after the occurrence of the event or events. Therefore, it is common to use the term "prophylaxis" as distinct from "treatment" to encompass both "preventing" and "suppressing" as defined herein. The term "protection," as used herein, is meant to include "prophylaxis." The "protection" provided need not be absolute, i.e., the disease need not be totally prevented or eradicated, provided that there is a statistically significant improvement relative to a control population. Protection can be limited to mitigating the severity or rapidity of onset of symptoms of the disease. See, e.g., Berkow et al, eds., *The Merck Manual*, 16th edition, Merck and Co., Rahway, N.J., 1992; Goodman *et al.*, eds., *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 8th edition, Pergamon Press, Inc., Elmsford, N.Y., (1990); *Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics*, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, MD (1987); Ebadi, *Pharmacology*, Little, Brown and Co., Boston, (1985); Osol et al., eds., *Remington's Pharmaceutical Sciences*, 17th edition, Mack Publishing Co., Easton, PA. (1990); Katzung, *Basic and Clinical Pharmacology*, Appleton and Lange, Norwalk, Conn, (1992), which references are entirely incorporated herein by reference.

At least one compound or composition of the present invention can be administered by any means that achieve the intended purpose, using a pharmaceutical composition as previously described.

For example, administration can be by various parenteral routes such as subcutaneous, intravenous, intramuscular, intraperitoneal, intranasal, intracranial, or buccal routes. Alternatively, or concurrently, administration can be by the oral route. Parenteral administration can be by bolus injection or by gradual perfusion over time.

It is understood that the dosage of a pharmaceutical compound or composition of the present invention administered *in vivo* or *in vitro* will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the pharmaceutical effect desired. The ranges of effective doses provided herein are not intended to be limiting and represent preferred dose ranges. However, the most preferred dosage will be tailored to the individual subject, as is understood and determinable by one skilled in the relevant arts. See, e.g., Berkow, *infra*, Goodman, *infra*, Avery, *infra* and Katzung, *infra*, which are entirely incorporated herein by reference, including all references cited therein.

The total dose required for each treatment can be administered by multiple doses or in a single dose. The pharmaceutical compound or composition can be administered alone or in conjunction with other diagnostics and/or pharmaceuticals directed to the pathology, or directed to other symptoms of the pathology.

Effective amounts of a pharmaceutical compound or composition of the present invention are from about 0.001 μg to about 100 mg/kg body weight, administered at intervals of 4-72 hours, for a period of 2 hours to 5 years, and/or any range or value therein, such as 0.000001-0.0001, 0.0001-0.01, 0.01-1.0, 1-10, 10-50 and 50-100, 0.000001-0.00001, 0.00001-0.0001, 0.0001-0.001, 0.001-0.01, 0.01-0.1, 0.1-1.0, 1.0-10, 5-10, 10-20, 20-50 and 50-100 mg/kg, at intervals of 1-2, 2-4, 4-6, 6-8, 8-10, 10-12, 12-14, 14-16, 16-18, 18-20, 20-22, 22-24, 24-26, 26-28, 28-30, 30-32, 32-34, 34-36, 36-40, 40-44, 44-48, 48-52, 52-56, 56-60, 60-64, 64-68, 68-72 hours, for a period of 0.6, 0.8, 1.0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32,

34, 36, 38, 40, 42, 44, 46, 48, 50, 60, 70, 80, 90, 100 days, or 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24, 28, 32, 36, 40, 44, 48, 52 and/or more weeks, and/or 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 30, 36, 40, 50, and/or 60 years, or any range or value therein.

The recipients of administration of compounds and/or compositions of the present invention can be any vertebrate animal, such as mammals, birds, bony fish, frogs and toads. Among mammals, the preferred recipients are mammals of the Orders Primata (including humans, apes and monkeys), Arteriodactyla (including horses, goats, cows, sheep, pigs), Rodenta (including mice, rats, rabbits, and hamsters), and Carnivora (including cats, and dogs). Among birds, the preferred recipients are turkeys, chickens and other members of the same order. The most preferred recipients are humans.

The methods provided by the present invention for inducing the survival and/or mitosis of NPCs with a PF, TF or TF-neurological agent can be used *in vivo*, e.g., increasing the supply of cells capable of differentiating into mature neurons.

Further, neurodegenerative diseases or pathologies can be treated by methods and compositions of the present invention. It is contemplated by the present invention that methods provided for inducing the proliferation of neuronal precursor cells as described above can be applied to treat neuronal disorders resulting in neuronal cell deficiencies. Neuronal deficiencies can be caused by physical damage to the brain such as that caused by trauma, surgery, etc. Neuronal deficiencies can also be caused by infection, nutritional deficiencies, and exposure to toxic agents. In addition, neuronal deficiencies can be caused by neurodegenerative disorders such as stroke, Alzheimer's disease, Parkinson's disease, Huntington's Chorea, familial dysautonomia, Amyotrophic Lateral Sclerosis (ALS), olivopontocerebellar atrophy, spinal muscular atrophy, hereditary sensorimotor neuropathy and static encephalopathy (i.e., cerebral palsy).

Neurodegenerative disorders which can be treated according to a method of the present invention include, but are not limited to, the following:

demyelinating diseases, such as multiple sclerosis and acute transverse myelitis;

extrapyramidal and cerebellar disorders, such as lesions of the corticospinal system;

disorders of the basal ganglia or cerebellar disorders;

hyperkinetic movement disorders such as Huntington's Chorea and senile chorea;

drug-induced movement disorders, such as those induced by drugs which block CNS dopamine receptors;

hypokinetic movement disorders, such as Parkinson's disease;

progressive supra-nucleo palsy;

structural lesions of the cerebellum;

spinocerebellar degenerations, such as spinal ataxia, Friedreich's ataxia, cerebellar cortical degenerations, multiple systems degenerations (Mencel, Dejerine-Thomas sporadic or recessive disorder, Shi-Drager, and Machado-Joseph disease);

systemic disorders (Refsum's disease, abetalipoproteinmia, ataxia telangiectasia, and mitochondrial multi-system disorder);

disorders of the motor unit, such as neurogenic muscular atrophies (anterior horn cell degeneration, such as amyotrophic lateral sclerosis (ALS), infantile spinal muscular atrophy and juvenile spinal muscular atrophy);

Alzheimer's disease;

Down's Syndrome in middle age;

Diffuse Lewy body disease;

Senile Dementia of Lewy body type;

Wernicke-Korsakoff syndrome;

the effects of chronic alcoholism;

Creutzfeldt-Jakob disease;

Subacute sclerosing panencephalitis

Hallerrorden-Spatz disease;

Dementia pugilistica;

neurological developmental diseases, such as those due to premature delivery or cocaine addiction. Additionally, trauma of the central nervous system can similarly be treated by a method according to the present invention. Non-limiting examples are trauma of the central nervous system, include head injury, postconcussion syndrome and spinal cord injury. See, e.g., Berkow *et al.*, eds., *The Merck Manual*, 16th edition, Merck and Co., Rahway, NJ (1992); Goodman *et al.*, eds., *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 8th edition, Pergamon Press, Inc., Elmsford, NY (1990); *Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics*, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, MD (1987); Katzung, ed., *Basic and Clinical Pharmacology*, Fifth Edition, Appleton and Lange, Norwalk, CT (1992), which references, and references cited therein, are entirely incorporated herein by reference for preparation and administration of pharmaceutical compositions.

The methods of the present invention may be applied in two basic ways to treat neuronal deficiencies. First, neuronal precursor cultures can be propagated according to the methods of the present invention and then grafted into the portion of the brain incurring the neuronal deficit (i.e., cell therapy). Placed in this environment, the precursors can then passively differentiate into replenishing neurons, or they can be induced to differentiate.

Neuronal precursors exhibit plasticity with regard to their capacity to develop into different neuronal types, depending upon the environment they are placed in (Purves & Lichtman, *Principles of Neural Development*, Sinauer Associates Inc. Publishers, Sunderland, MA (1985); O'Leary, *Trends in Neurosci. 12*:400-406 (1989); Renfranz *et al.*, *Cell 66*:713-729 (1991); Snyder *et al.*, *Cell 68*:33-51 (1992)). Thus, it is contemplated in the present invention that a particular neuronal precursor cell can be used, not only to replenish neurons it typically differentiates into, but can also be used to replenish other neuronal cell types which can differentiate from, as differentiation products, under the appropriate environmental conditions. In

particular, it is contemplated that sympathetic precursors, cerebellar granule precursors, and telencephalic precursors from the embryonic cortex and striatum can be used to replenish a variety of neuronal cell types including neuronal cells from the cerebral cortex, basal ganglia, brain stem, spinal cord, retina and peripheral nervous system.

Having now generally described the present invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and is not intended to be limiting of the present invention.

Examples

Example 1: In Vivo Regulation of Cerebellar Granule Cell Neurogenesis by bFGF

Background

While diverse neuronal precursor populations have been examined in culture, little is known about mechanisms regulating neuroblast proliferation *in vivo*. Precursors of cerebellar granule cells, a useful model culture system, are known to respond to a number of growth factors, including insulin growth factors (IGF's), epidermal growth factor (EGF), vasoactive intestinal polypeptide (VIP), tumor necrosis factor (TNF) and bFGF (DiCicco-Bloom *et al., Society for Neuroscience Abstracts XV*:328 (1989); Chen *et al., Society for Neuroscience Abstracts XVI*:803 (1990); Cohen. *et al., Society for Neuroscience Abstracts XVI*:804 (1990); Gao *et al., Neuron* 6:705-715 (1991)), which influence neuroblast mitosis and/or survival. We now report that bFGF specifically stimulates cerebellar granule neuroblast DNA synthesis in the postnatal day 1 rat.

Results

To define effects in vivo, rats were injected subcutaneously (sc) with growth factors or saline vehicle every 2 hours for up to 5 doses, and received 4-5 μCi/g of (^{3}H)thymidine sc 2 hours prior to sacrifice. bFGF (5 ng/g per dose) elicited a 28.7 $\pm$ 10% ($p < 0.016$) increase in (^{3}H)thymidine incorporation in whole cerebellar homogenates at 10 hours, suggesting that the factor influenced ongoing neurogenesis.

To define response requirements, different dose schedules were employed. Remarkably, a single dose of bFGF (5 ng/g) elicited a similar increase in DNA synthesis (50.3 $\pm$ 7.6%; $p < 0.01$) at 8 hours. In contrast, five injections of EGF (0.25 μg/g/dose), sufficient to elicit peripheral edema, did not affect incorporation, suggesting that bFGF actions were specific.

To begin defining responsive populations, cerebellar granule cells were isolated by centrifugation following *in vivo* growth factor and (^{3}H)thymidine administration. There was a striking four-fold increase in incorporation (per 1-3 x 10^5 cells) following bFGF treatment, indicating that granule neuroblasts comprised a major responsive population. These observations raise the possibility that bFGF plays a critical role in cerebellar granule neurogenesis *in vivo*, though underlying cellular and molecular mechanisms remain to be defined.

Example 2: Mitogenicity of bFGF on Cerebellar Granule Cells in Culture

Background

To define responsiveness of specific brain cell populations to compounds which exhibit in vivo mitogenicity, bFGF was tested for its effect on cerebellar granule cells.

Materials and Methods

Experimental Animals. Time-mated, pregnant rats and mice and postnatal pups are obtained and housed in an NIH approved and institutionally operated animal care facility. Midnight is considered the time of conception and the day following mating is designated as day 0.5 of gestation. The day following birth is considered postnatal day 1 (P1).

Dissection. Superior cervical ganglia (SCG) from gestational day 15.5 (E15.5) embryos are obtained after CO_2 euthanasia as reported (DiCicco-Bloom *et al.*, *Proc. Natl. Acad. Sci. USA 85*:4066-4070 (1988)). To obtain cerebella, following CO_2 euthanasia and cervical section, the cranial vault and underlying brain is obtained by section extending from ventral to the medulla to the level of the orbits. The cerebellum is dissected free of dorsal brain and pia-arachnoid in phosphate-buffered saline (PBS) and the cerebellar cortex is separated from the underlying deep nuclei and white matter.

Dissociation of Cerebellar Granule Neuroblasts. The following techniques have been reported (Hatten, *J. Cell. Biol. 100*: 384-396 (1985); Hatten *et al.*, *Devel. Biol. 125*:280-289 (1988)) and are briefly summarized. Cerebellar cortices are incubated in trypsin/DNase (Worthington) (10 mg/1 mg/ml, respectively) - PBS solution for 3 min. at 37°C and triturated with fire-polished Pasteur pipettes in DNAse (0.5 mg/ml) solution. After pelleting cells and resuspension in PBS, the dissociate is filtered through a 30 micron Nitex filter (Tetko), pelleted, resuspended and overlayed on a 35%/60% Percoll (Pharmacia, from Sigma) in PBS step-gradient. After centrifugation at 3200 rpm for 10 min., large cells from the PBS/35% interface are discarded and small cells are collected from the 35%/60% interface, washed in PBS, plated in defined medium for 1 hour on poly-D-lysine-coated (0.1 mg/ml) dishes to remove highly adhesive cells, collected by gentle pipetting, counted and distributed to culture dishes (DiCicco-Bloom *et al.*, *Soc. Neurosci. XV*:328 (1989); Hatten, *J. Cell. Biol.100*:384-396 (1985)).

Culture Medium and Dishes. Derived from N2 medium of Bottenstein and Sato, serum-free defined medium is formulated and prepared as previously reported by the author (DiCicco-Bloom *et al.*, *Proc. Natl. Acad. Sci. USA 85*:4066-4070 (1988); DiCicco-Bloom *et al.*, *J. Cell Biol. 110*(6):2073-2086 (1990)), employing bovine serum albumin (BSA) at 10 mg/ml for SCG neuroblasts and 5 mg/ml for granule cells. Plastic 35 mm tissue culture dishes or 24 multiwell plates are prepared as previously reported (DiCicco-Bloom *et al.*, *J. Cell Biol. 110*(6):2073-2086 (1990); Pincus *et al.*, *Nature 343*:564-567 (1990)). Cultures are maintained for 1-4 days in a CO_2 incubator (37°C) adjusted to medium pH is 7.4 (DiCicco-Bloom *et al.*, *Proc. Natl. Acad. Sci. USA 85*:4066-4070 (1988); DiCicco-Bloom *et al.*, *J. Cell Biol. 110*(6):2073-2086 (1990)).

DNA Synthesis Assay. DNA synthesis was assayed as previously reported (DiCicco-Bloom *et al.*, *Proc. Natl. Acad. Sci. USA 85*:4066-4070 (1988); DiCicco-Bloom *et al.*, *J. Cell Biol. 110*(6):2073-2086 (1990); Pincus *et al.*, *Nature 343*:564-567 (1990)). After 24 hrs. incubation of cells in 24 multiwells with (^{3}H)thymidine (^{3}H)dT) 1μCi/ml medium, medium is aspirated and cells are exposed to trypsin 0.25%/EDTA 0.5 mM for 20 min., at 37°C, and collected on glass fiber filters. Filters are exhaustively washed with dH_2O, dried and counted in scintillant. Assay characterization indicates linearity with time and cell number up to 100,000 cells/well.

Immunocytochemistry. Cultured cells are rinsed in PBS and fixed in 2-4% paraformalydehyde for 20 min. or 5% acetic acid/95% ethanol for 5 min., according to specific antigen requirements, rinsed with PBS and incubated with primary antibodies according to previously published methods. Secondary antibody procedures will be according to the indirect immunofluorescence method of Coons or the immunoperoxidase staining method employing Vector Labs ABC Kit, as previously reported by this laboratory (DiCicco-Bloom *et al.*, *Proc. Natl. Acad. Sci. USA 85*:4066-4070 (1988); DiCicco-Bloom *et al.*, *J. Cell Biol. 110*(6):2073-2086 (1990)). Antibodies to specific antigens and methods are: neurofilament 160 kd

subunit, vimentin and fibronectin antibodies (Boehringer Mannhein; Bennett, *Curr. Topics Devel. Biol. 21*:151-183 (1987); Trojanowski *et al.*, *J. Neurosci. 6*:650-660 (1987)), neuron-specific enolase (Polysciences; as per catalogue), monoclonal antibodies 4D7 (TAG-1), 96A1 (L1), and 5A5 (NCAM) (Dodd *et al.*, *Neuron 1*:105-116 (1988); Yamamoto *et al.*, *J. Neurosci. 6*:3576-3594 (1986)), S-100 and GFAP (Dako) as previously described (DiCicco-Bloom *et al.*, *Proc. Natl. Acad. Sci. USA 85*:4066-4070 (1988)), tyrosine hydroxylase (DiCicco-Bloom *supra*, (1988); DiCicco-Bloom *et al.*, *J. Cell Biol. 110*(6):2073-2086 (1990)). Controls can include preimmune sera, deletion of primary antibody or preincubation with available antigen accordingly. In addition, nonneuronal tissues can be employed as negative and mature neurons as positive controls as indicated.

Autoradiography. Cultures previously incubated with (^{3}H)dT for 2 or 24 hrs. are processed by routine histology or by combined immunocytochemistry and autoradiography as previously described (DiCicco-Bloom, *supra*, (1988); DiCicco-Bloom *et al.*, *J. Cell Biol. 110*(6):2073-2086 (1990)). Cells exhibiting dense silver grains specifically localized to the nucleus, a marker for DNA synthesis, are considered to be in the mitotic cycle. Caveats concerning this interpretation have been discussed previously (DiCicco-Bloom, *supra*, (1988); DiCicco-Bloom *et al.*, *J. Cell Biol. 110*(6):2073-2086 (1990)). Moreover, addition to culture of the specific inhibitor of replicative DNA alpha-polymerase, Aphidicolin, prevents nuclear labeling.

Labeling Index (LI). The percentage of cultured cells labeled with dense nuclear silver grains referred to as the LI (DiCicco-Bloom *et al.*, *Proc. Natl. Acad. Sci. USA 85*:4066-4070 (1988)). For each experimental group consisting of 3 culture dishes, 100 cells in 3 to 4 random, non-overlapping areas are scored at 250 × magnification for silver grains on each dish, until 1000 are counted. These 10 determinations are analyzed statistically as below.

For *in vivo* labeling, bromodeoxyuridine (BUDR) is employed. Following i.p. injection of BUDR 50-100 μg/gm weight, animals are sacrificed 30 min. later, fixed in 70% ethanol and processed according to published protocols (Nowakowski *et al.*, *J. Neurocytology 18*:311-318 (1989)) employing Vectastain ABC kits (Vector Labs). However, for *in situ* hybridization, tissues will be fixed in 4% paraformaldehyde, necessitating pretreatment of sections with 0.1% trypsin for 30 min. prior to DNA denaturation (Nowakowski *et al.*, *J. Neurocytology 18*:311-318 (1989)).

Cell Counting and Morphometry. Cell numbers in groups of 3 to 4 living cultures are assayed employing an inverted, phase-microscope with graduated stage as previously reported (DiCicco-Bloom *et al.*, *Proc. Natl. Acad. Sci. USA 85*:4066-4070 (1988); DiCicco-Bloom *et al.*, *J. Cell Biol. 110*(6):2073-2086 (1990)). Neuritic process elaboration is assessed as an index of morphological differentiation. Initiation of neurite outgrowth is assessed at 6-10 hrs., measuring processes extending greater than two cell-soma diameters.

Results

bFGF demonstrated mitogenic effects on cerebellar granule cells in culture. Figure 2 illustrates that increased tritiated thymidine incorporation was exhibited when the granule cells were treated with bFGF. A control for mitogenicity was also performed (*see*, e.g., Figure 2, the bar labeled "Con"). bFGF demonstrated a more pronounced mitogenic effect than that seen for the control.

These data demonstrate that cells which are sequestered within the blood brain barrier *in vivo* are responsive to the mitogenic effects of bFGF *in vitro*. These results indicate that the cultured cells possess receptors for bFGF.

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the present invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

What Is Claimed Is:

1. A method for transporting a neurological agent across the blood-brain barrier of a mammal, comprising

administering to said mammal a conjugate comprising

(A) at least one neurological agent; linked to:

(B) at least one transport factor comprising at least one portion of a growth factor, said transport factor capable of effecting transport of said neurological agent across the blood brain barrier; and

(C) a pharmaceutically acceptable carrier or diluent.

2. A method according to claim 1, wherein said growth factor is selected from the group consisting of a fibroblast growth factor (FGF), an epidermal growth factor (EGF), an insulin-like growth factor (IGF), an insulin, a vasoactive intestinal peptide (VIP), a pituitary adenylate cyclase activating polypeptide (PACAP), a tumor necrosis factor (TNF), a ciliary neurotrophic factor (CNTF), a brain derived neurotrophic factor (BDNF), a neurotrophin-3 (NT3), a somatostatin and an interferon (IFN).

3. A method according to claim 1, wherein said growth factor is selected from the group consisting of an IFNα, an IFNβ, a leukemia inhibitory factor (LIF), a nerve growth factor (NGF), a calcitonin gene related peptide (CGRP), a vasopressin, a bradykinin, a bombesin, a Substance P, an enkephelin and an interleukin.

4. A method according to claim 1, wherein said growth factor is a FGF.

5. A method according to claim 4, wherein said FGF binds an FGF receptor.

6. A method according to claim 4, wherein said FGF is at least one selected for the group consisting of basic FGF, acidic FGF, an hst/K-fgf gene product, FGF-6, KFG, FGF-5 and int-2.

7. A method according to claim 6, wherein said FGF is basic FGF.

8. A method according to claim 1, wherein said neurological agent is selected from the group consisting of a protein, a hormone, an antibiotic, a nucleotide, a nucleotide analogue, a drug, and a small molecule.

9. A method according to claim 8, wherein said protein is a growth factor.

10. A method according to claim 8, wherein said growth factor is selected from the group consisting of an epidermal growth factor (EGF), an insulin-like growth factor (IGF), an insulin, a vasoactive intestinal peptide (VIP), a pituitary adenylate cyclase activating polypeptide (PACAP), a tumor necrosis factor (TNF), a ciliary neurotrophic factor (CNTF), a brain derived neurotrophic factor (BDNF), a neurotrophin-3 (NT3), a somatostatin and an interferon (IFN).

11. A method according to claim 8, wherein said growth factor is selected from the group consisting of an IFNα, an IFNβ, a leukemia inhibitory factor (LIF), a nerve growth factor (NGF), a calcitonin gene related peptide (CGRP), a vasopressin, a bradykinin, a bombesin, a Substance P, an enkephelin and an interleukin.

12. A method according to claim 1, wherein said administering is intravenous administration.

13. A method according to claim 1 wherein said mammal is selected from the group consisting of a mouse, a rat, a rabbit and a human.

14. A method according to claim 13, wherein said mammal is human.

15. A conjugate composition, comprising

(A) at least one neurological agent; linked to:

(B) at least one transport factor comprising at least one portion of a growth factor, said transport factor capable of effecting transport of said neurological agent across the blood brain barrier; and

(C) a pharmaceutically acceptable carrier or diluent.

16. A conjugate composition according to claim 15, wherein said growth factor is selected from the group consisting of a fibroblast growth factor (FGF), an epidermal growth factor (EGF), an insulin-like growth factor (IGF), an insulin, a vasoactive intestinal peptide (VIP), a pituitary adenylate cyclase activating polypeptide (PACAP), a tumor necrosis factor (TNF), a ciliary neurotrophic factor (CNTF), a brain derived neurotrophic factor (BDNF), a neurotrophin-3 (NT3), a somatostatin and an interferon (IFN).

17. A conjugate composition according to claim 15, wherein said growth factor is selected from the group consisting of an IFNα, an IFNβ, a leukemia inhibitory factor (LIF), a nerve growth factor (NGF), a calcitonin gene related peptide (CGRP), a vasopressin, a bradykinin, a bombesin, a Substance P, an enkephelin and an interleukin.

18. A conjugate composition according to claim 16, wherein said growth factor is a FGF.

19. A conjugate composition according to claim 18, wherein said FGF binds an FGF receptor.

20. A conjugate composition according to claim 18, wherein said FGF is at least one selected for the group consisting of basic FGF, acidic FGF, an hst/K-fgf gene product, FGF-6, KFG, FGF-5 and int-2.

21. A conjugate composition according to claim 18, wherein said FGF is basic FGF.

22. A conjugate composition according to claim 15, wherein said neurological agent is selected from the group consisting of a protein, a peptide, a hormone, an antibiotic, a nucleotides, a nucleotide analogue, a drug, and a small molecule.

23. A conjugate composition according to claim 22, wherein said protein is a growth factor.

24. A conjugate compostion according to claim 23, wherein said growth factor is selected from the group consisting of an epidermal growth factor (EGF), an insulin-like growth factor (IGF), an insulin, a vasoactive intestinal peptide (VIP), a pituitary adenylate cyclase activating polypeptide (PACAP), a tumor necrosis factor (TNF), a ciliary neurotrophic factor (CNTF), a brain derived neurotrophic factor (BDNF), a neurotrophin-3 (NT3), a somatostatin and an interferon (IFN).

25. A conjugate composition according to claim 23, wherein said growth factor is selected from the group consisting of an IFNα, an IFNβ, a leukemia inhibitory factor (LIF), a nerve growth factor (NGF), a calcitonin gene related peptide (CGRP), a vasopressin, a bradykinin, a bombesin, a Substance P, an enkephelin and an interleukin.

26. A process for processing a composition for the treatment of a neurological disorder, comprising combining a neurological agent and a transport factor according to claim 15.

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ATG GCA GCC GGG AGC ATC ACC ACG CTG CCC GCC TTG CCC GAG GAT GGC
Met Ala Ala Gly Ser Ile Thr Thr Leu Pro Ala Leu Pro Glu Asp Gly
 1               5                  10                  15

GGC AGC GGC GCC TTC CCG CCC GGC CAC TTC AAG GAC CCC AAG CGG CTG
Gly Ser Gly Ala Phe Pro Pro Gly His Phe Lys Asp Pro Lys Arg Leu
            20                  25                  30

TAC TGC AAA AAC GGG GGC TTC TTC CTG CGC ATC CAC CCC GAC GGC CGA
Tyr Cys Lys Asn Gly Gly Phe Phe Leu Arg Ile His Pro Asp Gly Arg
        35                  40                  45

GTT GAC GGG GTC CGG GAG AAG AGC GAC CCT CAC ATC AAG CTA CAA CTT
Val Asp Gly Val Arg Glu Lys Ser Asp Pro His Ile Lys Leu Gln Leu
    50                  55                  60

CAA GCA GAA GAG AGA GGA GTT GTG TCT ATC AAA GGA GTG TGT GCT AAC
Gln Ala Glu Glu Arg Gly Val Val Ser Ile Lys Gly Val Cys Ala Asn
65                  70                  75                  80

CGT TAC CTG GCT ATG AAG GAA GAT GGA AGA TTA CTG GCT TCT AAA TGT
Arg Tyr Leu Ala Met Lys Glu Asp Gly Arg Leu Leu Ala Ser Lys Cys
                85                  90                  95

GTT ACG GAT GAG TGT TTC TTT TTT GAA CGA TTG GAA TCT AAT AAC TAC
Val Thr Asp Glu Cys Phe Phe Phe Glu Arg Leu Glu Ser Asn Asn Tyr
            100                 105                 110

AAT ACT TAC CGG TCA AGG AAA TAC ACC AGT TGG TAT GTG GCA TTG AAA
Asn Thr Tyr Arg Ser Arg Lys Tyr Thr Ser Trp Tyr Val Ala Leu Lys
        115                 120                 125

CGA ACT GGG CAG TAT AAA CTT GGA TCC AAA ACA GGA CCT GGG CAG AAA
Arg Thr Gly Gln Tyr Lys Leu Gly Ser Lys Thr Gly Pro Gly Gln Lys
    130                 135                 140

GCT ATA CTT TTT CTT CCA ATG TCT GCT AAG AGC TGA
Ala Ile Leu Phe Leu Pro Met Ser Ala Lys Ser Ter
145                 150                 155
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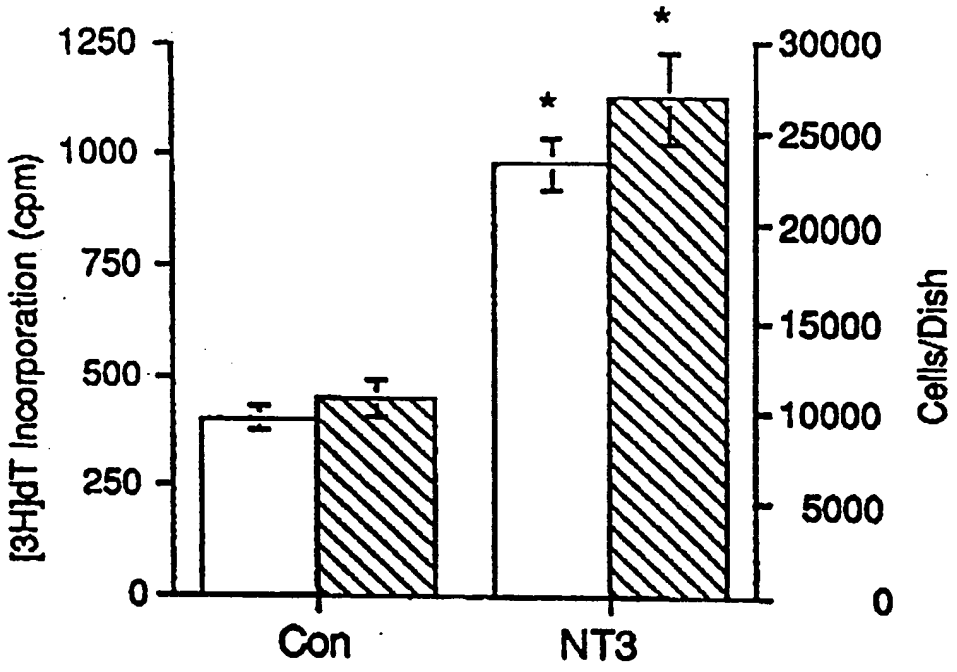
FIGURE 1


1250
1000
750
500
250
0
[3H]dT Incorporation (cpm)
30000
25000
20000
15000
10000
5000
0
Cells/Dish
*
*
Con
NT3

- FIGURE 2 -

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09155

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.4; 435/69.1, 69.4, 69.51, 69.7; 514/2, 3, 4; 530/300, 302, 303, 311, 350, 351, 399

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 4,801,571 (PARDRIDGE ET AL.) 31 JANUARY 1989, col. 2, lines 22-58; col. 3, line 4 - col. 5, line 30; abstract; claims 1-19.	1-2, 8-10, 12-16, 22-24, 26 --- 3-7, 11, 17-21, 25
A,P	Molecular Brain Research, Volume 22, issued 1994, R. Boado et al., "Enhanced expression of the blood-brain barrier GLUT1 glucose transporter gene by brain-derived factors", pages 259-267, especially Table 1; Figure 5; page 265; abstract.	1-10, 12-16, 18-24, 26
A	Biotherapy, Volume 3, issued 1991, S. Shin, "Chimeric antibody: Potential applications for drug delivery and immunotherapy", pages 43-53, especially page 50 and abstract.	1-26

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 OCTOBER 1994	Date of mailing of the international search report DEC 28 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Arie M. Michelsohn, Ph.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Intern. ..onal application No.
PCT/US94/09155

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Cell and Tissue Research, Volume 241, issued 1985, K. Nave et al., "Epidermal growth factor does not cross the blood-brain barrier", pages 453-457, especially abstract; page 455.	1-26
X,P ---- Y,P	EP, A, 0,599,303 (FUKUTA ET AL.) 01 June 1994, entire document.	1-2, 8-10, 12-16, 22-24, 26 ---------- 3-7, 11, 17-21, 25
Y	US, A, 5,154,924 (FRIDEN) 13 October 1992, col. 2, lines 28-69; col. 5, lines 36-66; claims 1, 8, 11; abstract.	1-26
A,P	US, A, 5,254,342 (SHEN ET AL.) 19 October 1993, entire document, especially col. 10, lines 13-38; claims 1-2; abstract.	1-26
Y	WO, A, 91/14696 (LATHAM ET AL.) 03 October 1991, abstract; pages 1-18; claims 1-85, 108-113; 129-131	1, 8, 12-15, 22, 26
A	WO, A, 91/04014 (COLLINS ET AL.) 04 April 1991, entire document.	1-26
A	Endocrine Reviews, Volume 7, Number 3, issued 1986, W.M. Pardridge, "Receptor-mediated peptide transport through the blood-brain barrier", pages 314-330.	1-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09155

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 38/00, 38/02, 38/08, 38/11, 38/21, 38/22, 38/28, 47/48; C07K 2/00, 7/00, 7/16, 7/18, 14/00, 14/62, 14/70, 14/585, 14/655, 14/675, 17/00, 17/02, 17/06, 19/00; C12N 15/00, 15/09, 15/12, 15/16, 15/17, 15/18, 15/19, 15/21, 15/28; C12P 21/00, 21/02

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/85.4; 435/69.1, 69.4, 69.51, 69.7; 514/2, 3, 4; 530/300, 302, 303, 311, 350, 351, 399

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG: files BIOSIS, MEDLINE, WORLD PATENT INDEX, BIOTECH ABSTRACTS, CURRENT BIOTECH ABSTRACTS, CHEMICAL ABSTRACTS
search terms: growth factor, conjugate/fusion/chimera, blood brain barrier, fgf